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### Characterization of the molecular and electrophysiological properties of the T-Type calcium channel in human myometrium

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1     **Characterization of the molecular and electrophysiological properties of the T-Type**  
2                             **calcium channel in human myometrium.**

3  
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1   **Abstract:**

2   Rises in intracellular calcium are essential for contraction of human myometrial smooth  
3   muscle (HMSM) and hence parturition. The T-type calcium channel may play a role in  
4   this process. The aim was to investigate the role of the T-Type calcium channel in  
5   HMSM by characterizing mRNA expression, protein localization, electrophysiological  
6   properties and function of the channel subunits  $\text{Ca}_v3.1(\alpha1\text{G})$ ,  $\text{Ca}_v3.2(\alpha1\text{H})$ , and  
7    $\text{Ca}_v3.3(\alpha1\text{I})$ . QRT-PCR, immuno-histochemistry, electrophysiology and *invitro*  
8   contractility were performed on human myometrial samples from term, preterm, labour  
9   and not in labour. QRT-PCR analysis of  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$  and  $\text{Ca}_v3.3$  demonstrated  
10   expression of  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  with no significant change ( $P>0.05$ ) associated with  
11   gestation or labour status. Immuno-histochemistry localized  $\text{Ca}_v3.1$  to myometrial and  
12   vascular smooth muscle cells whilst  $\text{Ca}_v3.2$  localized to vascular endothelial cells and  
13   invading leukocytes. Voltage clamp studies demonstrated a T-type current in 55% of  
14   cells. Nickel block of T-type current was voltage sensitive ( $\text{IC}_{50}$  of  $118.57\pm68.9 \mu\text{M}$  at -  
15   30mV). Activation and inactivation curves of  $\text{I}_{\text{Ca}}$  currents in cells expressing T-type  
16   channels overlapped demonstrating steady state window currents at the resting membrane  
17   potential of myometrium at term. Current clamp analysis demonstrated that  
18   hyperpolarizing pulses to a membrane potential greater than -80mV elicited rebound  
19   calcium spikes that were blocked reversibly by 100 $\mu\text{M}$  nickel. Contractility studies  
20   demonstrated a reversible decrease in contraction frequency during application of 100 $\mu\text{M}$   
21   nickel ( $P<0.05$ ). We conclude that the primary T-type subunit expressed in some MSMCs  
22   is  $\text{Ca}_v3.1$ . We found that application of 100  $\mu\text{M}$  nickel to spontaneously contracting  
23   human myometrium reversibly slows contraction frequency.

## 1    **Introduction**

2    The contractile activity of the uterus undergoes major modifications during the transition  
3    from the relative quiescence of the majority of gestation to the highly coordinated  
4    contractions of delivery. The transition of the myometrium into a contractile phenotype  
5    has been termed the “activation”(Challis *et al.*, 2000) or “preparatory phase”(Garfield *et*  
6    *al.*, 1998). This process, under the stimulation of oestrogen and a reduction in the  
7    influence of progesterone involves molecular changes that manifest in an increase in  
8    contraction frequency and a depolarized resting membrane potential in term  
9    myometrium(Parkington *et al.*, 1999a) when compared to mid-gestation.

10    Although much research has been dedicated to the mechanism by which the sex steroids  
11    mediate their influence on myometrium little is known about the physiological  
12    mechanism or the molecular identity of currents that manifest spontaneous contractions at  
13    term. Some investigations have explored the role of calcium activated  $K^+$  channels  
14    (Anwer *et al.*, 1993; Khan *et al.*, 1993; Perez *et al.*, 1993; Khan *et al.*, 1997) and voltage  
15    activated  $K^+$  channels (Knock *et al.*, 1999) in control of resting membrane potential and  
16    repolarisation. However the molecular identity or physiology of a pacemaker current  
17    remains elusive.

18    What is clear is that control of resting membrane potential and rises in intracellular  
19    calcium are essential for contraction in human myometrial smooth muscle  
20    (MSM)(Parkington *et al.*, 1999b). Calcium is not only an important second messenger  
21    for the generation of force via myosin light chain kinase, but also depolarizes the plasma  
22    membrane allowing for activation of other voltage-dependent ion channels. This latter

1 property is an important function for T-type or low voltage activated (LVA) calcium  
2 channels, which are responsible for generating low threshold spikes that in neurons lead  
3 to burst firing and oscillatory behavior(Kim *et al.*, 2001). The sub-family of T-type  
4 calcium channels currently comprises of 3 differing alpha subunits termed  $Ca_v3.1(\alpha1G)$ ,  
5  $Ca_v3.2(\alpha1H)$ , and  $Ca_v3.3(\alpha1I)$ (Cribbs *et al.*, 1998; Perez-Reyes *et al.*, 1998; Lee *et al.*,  
6 1999a; Monteil *et al.*, 2000) .  $Ca_v3.1$  and  $Ca_v3.2$  demonstrate similar activation and  
7 inactivation kinetics but can be differentiated by sensitivity to nickel and recovery from  
8 inactivation.  $Ca_v3.3$  by contrast is easily distinguishable by its slower activation and  
9 inactivation kinetics.

10 In a recent molecular study in the rat it was demonstrated that both  $Ca_v3.1$  and  $Ca_v3.2$   
11 were expressed in circular and longitudinal layers of myometrium and that the relative  
12 expression profile of these channels differed, dependent on gestational age, layer and  
13 subunit(Ohkubo *et al.*, 2005b).

14 It has previously been demonstrated in electrophysiological studies that a T-type like  
15 current is present in human myometrium (Young *et al.*, 1993) and that it is larger in  
16 magnitude than the more extensively investigated L-Type current. In a recent study it  
17 was demonstrated that administration of Mibefradil, a partially selective T-type inhibitor,  
18 reduced the force generated during a contraction whilst decreasing the magnitude of the  
19 initiation spike of tissue level electrical activity (Young & Zhang, 2005). This suggests  
20 that the T-type calcium channel may be involved in the initiation of action potentials in  
21 uterine smooth muscle. We sought to investigate the role of the T-Type calcium channel

- 1 in human myometrium by characterizing the expression, electrophysiological properties
- 2 and function of channel subunits  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$ , and  $\text{Ca}_v3.3$ .

## 1    **Subjects and Methods**

### 2    *Subject Criteria and Selection*

3    All procedures were conducted within the guidelines of “The Declaration of Helsinki”  
4    and were subject to local ethical approval (REC-05/Q2802/107). Prior to surgery,  
5    informed written consent for sample collection was obtained. Subjects were recruited  
6    into two groups, spontaneous labour (LAB) and elective cesarean section not in labour  
7    (NIL) between 32-40wks gestation. Term was defined as >37 completed weeks gestation  
8    and pre-term labour defined as <37 completed weeks. The LAB group was undergoing  
9    cesarean section for reasons of presumed fetal distress. LAB was defined as regular  
10    contractions (<3mins apart), membrane rupture, and cervical dilatation (>2cm) with no  
11    augmentation.

### 12    *Sample collection*

13    At cesarean section, samples were collected before Syntocin administration by knife  
14    biopsy from the lower uterine segment incision. Samples were washed briefly in saline  
15    and flash-frozen in liquid nitrogen for mRNA/immuno-histochemistry. Samples for cell  
16    isolation were placed in ice cold modified Krebs-Henseleit solution (see below) and  
17    utilized the same day.

### 18    *Solutions*

19    Fresh samples were stored in ice cold modified Krebs-Henseleit (m-KHS) solution  
20    containing (mmol/L): NaCl, 133; KCl, 4.7; TES, 10; Glucose, 11.1; MgSO<sub>4</sub>, 1.2;



1 KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; adjusted to pH 7.4 at 25°C with 5M NaOH. Krebs-Henseleit  
 2 (KHS) solution contained : NaCl, 118; NaHCO<sub>3</sub>, 25; KCl, 4.7; Glucose, 11.1 MgSO<sub>4</sub>,  
 3 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 2.5; pH 7.4 was maintained by constant aeration with 5%  
 4 CO<sub>2</sub>/95% O<sub>2</sub> at 37°C. Ca<sup>2+</sup>-free Tyrode's solution contained : NaCl, 136; KCl, 5.4;  
 5 MgSO<sub>4</sub>, 1.0; NaH<sub>2</sub>PO<sub>4</sub>, 0.33; glucose, 5; HEPES, 10; adjusted to pH 7.4 at 25°C with 5M  
 6 NaOH. Digestion solution (DS) contained: Ca<sup>2+</sup>-free Tyrode's; Sigma type IX  
 7 collagenase, (1790IU/mg)1.25 mg ml<sup>-1</sup>; Sigma type IA collagenase, (535 IU/mg)  
 8 1.25 mg ml<sup>-1</sup>; Bovine Serum Albumin, 1 mg ml<sup>-1</sup>. Kraftbrühe (KB) solution was as  
 9 described in Klökner & Isenberg(Klockner & Isenberg, 1985). Ca<sup>2+</sup>-free physiological  
 10 salt solution (PSS) contained: NaCl, 130; KCl, 5; MgCl<sub>2</sub>, 1.2; HEPES, 10; and glucose,  
 11 10; adjusted to pH 7.4 at 25°C with 5M NaOH. The electrode (internal) solution for  
 12 voltage clamp contained: CsCl,135; MgCl<sub>2</sub> 2.5; MgATP, 5; HEPES, 10; and EGTA, 10;  
 13 adjusted to pH 7.2 with CsOH. The bath solution was composed of : NaCl,120; CsCl, 1.0;  
 14 tetraethylammonium chloride (TEA-Cl), 4.0; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 2.0; HEPES, 10.0; and  
 15 glucose, 10.0; the pH was adjusted to pH 7.4 with 5M NaOH. For current clamp  
 16 experiments bath solution was m-KHS. Electrode solution used in these experiments  
 17 contained: NaCl, 12; KCl, 40; K-Glutamate, 90; TES, 10;Na-Pyruvate, 1; MgSO<sub>4</sub>, 1;  
 18 EGTA, 0.2; CaCl<sub>2</sub>, 0.0803; pH 7.2. Nifedipine stock solution was 10 mmol/L in DMSO.  
 19 Nickel stock solution was 100mmol/L in water. Final DMSO concentrations were less  
 20 than 0.01%. All reagents were obtained from Sigma (Sigma-Aldrich Co., Dorset, UK)  
 21 unless otherwise stated.

## 22 *Cell Isolation*

1 Strips of myometrium (2×2×4~5 mm) were incubated for 30 min in Ca<sup>2+</sup>-free  
2 Tyrode's followed by 40~45 min at 37°C in DS. Digestion was terminated by  
3 dilution in pre-warmed Ca<sup>2+</sup>-free Tyrode's followed by centrifugation for 10 mins  
4 at 250g. Cells were dispersed by slow trituration through a wide bore fire polished  
5 glass pipette in KB solution. Single myometrial cells were filtered through a 200-  
6 µM gauze and stored in KB solution for at least 1 hour before experimental  
7 procedures. All experiments were performed within 6 hours after isolation.

8

### 9 *Electrophysiology*

#### 10 Voltage Clamp

11 A drop of myometrial cell suspension was placed in a cell chamber (1.0ml) onto a glass  
12 coverslip mounted on the stage of an inverted microscope (IX51, Olympus). After settling  
13 (approx 20min) cells were superfused with bath solution for 10 min at a rate of 1~2  
14 mL/min at room temperature. Transmembrane currents were recorded with an amplifier  
15 (Axopatch 200B; Axon Instruments). Patch pipettes were fabricated (MODEL P-87;  
16 Sutter Instruments, Novato, CA) from 1.5mm glass capillaries with a resistance of 2.0–  
17 4.0MΩ when filled with pipette solution. Liquid junction potential was zeroed prior to  
18 seal formation.

19 Following the formation of gigaseal, the membrane was ruptured by gentle suction  
20 obtaining the whole-cell voltage-clamp configuration. Membrane capacitance and series  
21 resistance were compensated after membrane rupture. Inward current was elicited by  
22 depolarising voltage steps at a frequency of 0.1Hz from a holding potential (HP) of

1 either -50mV or -80mV to +60mV in 10mV increments. Currents were filtered at 1 kHz  
2 and sampled at 2 kHz. Voltage protocols were delivered via a Digidata 1320 computer  
3 interface using pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). Passive  
4 leakage currents were subtracted using a Positive/Negative (P/N) protocol. All  
5 experiments were carried out at 22–24 °C.

## 6 Current clamp

7 Cells were treated similarly to voltage clamp experiments. The resting potential and  
8 action potentials were recorded using 4–6 M $\Omega$  pipettes connected to a headstage of a  
9 discontinuous voltage/current clamp amplifier (SEC-05, npi electronic GmbH, Tamm,  
10 Germany) operated in discontinuous current clamp mode. The current and voltage outputs  
11 of the amplifier were recorded and the current stimuli delivered via an ITC-18 computer  
12 interface (InstruTECH, Port Washington, NY, USA) controlled by PatchMaster software  
13 (HEKA Elektronik GmbH, Lambrecht, Germany). Liquid junction potential of 15 mV  
14 was subtracted from the obtained records. All experiments were carried out at 35 °C.

## 15 RNA analysis

16 RNA was isolated and reverse transcribed into cDNA for PCR analysis as described  
17 previously (Astle *et al.*, 2005). The expressions of r18s, Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3 were  
18 verified by real-time RT-PCR using the ABI PRISM 7700 Sequence Detection System  
19 (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. TaqMan  
20 gene expression assay primer/probe identification numbers were Ca<sub>v</sub>3.1  
21 (Hs00367969\_m1), Ca<sub>v</sub>3.2 (Hs00184168\_m1), and Ca<sub>v</sub>3.3 (Hs00184168\_m1) (Applied

1 Biosystems, Foster City, CA). Expression was normalized using the  $\Delta\Delta C_T$  method to  
2 r18s and non-pregnant myometrium. Amplification efficiency was determined over a  
3 linear cDNA titration for each probe set.

#### 4 *Immuno-histochemistry*

##### 5 Sample preparation

6 Frozen sections (8 $\mu$ m) of myometrium were briefly air dried and fixed for 30min in ice  
7 cold 4% paraformaldehyde in phosphate buffered saline. Isolated myocytes were plated  
8 onto cover slips in HBSS and placed overnight in a culture incubator. Plated cells were  
9 fixed for 5 min in ice cold 4% paraformaldehyde in phosphate buffered saline.

##### 10 Antibody incubation

11 Detection of Ca<sub>v</sub>3.1, Ca<sub>v</sub>3.2, and Ca<sub>v</sub>3.3 was performed according to manufacturer's  
12 instruction using a 1:200 dilution (Alomone labs, Jerusalem, Israel).

13 Secondary antibody (anti-rabbit Alexa 635) (Molecular Probes, Invitrogen Ltd, Paisley,  
14 UK) was used according to manufacturer's instruction using a 1:200 dilution. Positive  
15 staining was compared with secondary antibody alone. F-actin staining was determined  
16 by addition of Phalloidin-488 (Molecular Probes, Invitrogen Ltd, Paisley, UK) according  
17 to manufactures instruction

##### 18 Confocal Microscopy

19 After preparation, tissue sections or cells were examined using a Zeiss 510 META  
20 confocal microscope with X40 (NA 1.3, Oil DIC ) & X63 (NA 1.4, Oil DIC) objectives.  
21 Pinhole was set to 1 Airy unit and data was captured in 512x512 format.

##### 22 *Myometrial Contractility*

1 Contractility experiments were performed as described previously (Woodcock *et al.*,  
2 2006). Myometrial strips were allowed a 2 hour equilibration period to establish  
3 spontaneous contractions prior to addition of 100  $\mu\text{mol/L}$   $\text{NiCl}_2$ .

#### 4 *Data Analysis & Statistics*

5 All raw electrophysiological, expression and contractility data was imported directly into  
6 IGOR PRO v5 (Wavemetrics Inc, Portland, OR) for graphical and statistical analysis.  
7 Statistical significance was determined by one-way ANOVA (Newman Keuls test) for  
8 expression data and one-tailed unpaired student T-Test for contractility & voltage clamp.

9

## 10 **Results**

### 11 *Expression $\text{Ca}_v3$ alpha subunit mRNA*

12 Quantitative RT-PCR analysis of the expression of  $\text{Ca}_v3.1$ ,  $\text{Ca}_v3.2$  and  $\text{Ca}_v3.3$  in human  
13 myometrium demonstrated subunit-dependent differences. Expression of  $\text{Ca}_v3.1$  and  
14  $\text{Ca}_v3.2$  were detected in myometrial cDNA from all gestational ages whilst  $\text{Ca}_v3.3$   
15 remained below the limit of detection in all samples tested. The level of expression of  
16  $\text{Ca}_v3.1$  (Fig. 1A) and  $\text{Ca}_v3.2$  (Fig. 1B) demonstrated no significant change ( $P>0.05$ )  
17 between pre-term not in labour ( $n=9$ ), pre term labour ( $n=5$ ), term not in labour ( $n=12$ )  
18 and term labour ( $n=10$ ).

19

### 20 *Localization of $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ protein*

21 After determining that mRNA encoding  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  was expressed throughout  
22 gestation in myometrial samples we sought to establish the cell types in which the  
23 proteins were expressed. Immuno-histochemistry utilizing an antibody raised to a twenty

1 two amino acid epitope of the rat  $\text{Ca}_v3.1$  demonstrated specific binding in myometrial  
2 (Fig. 2A) and vascular smooth muscle cells (Fig. 2D). In contrast, antibody raised to a  
3 fifteen amino acid epitope of the rat  $\text{Ca}_v3.2$  demonstrated specific binding in vascular  
4 endothelial cells (Fig. 2E) and invading leukocytes (Fig. 2H). The precise localization of  
5  $\text{Ca}_v3.1$  was further investigated by high magnification confocal microscopy in dissociated  
6 myometrial smooth muscle cells (MSMC). Detailed scans demonstrated localization of  
7  $\text{Ca}_v3.1$  protein in discrete punctate regions of the plasma membrane and in the peri-  
8 nuclear area of the sarcoplasmic reticulum (Figs. 2J, K, L).

9

#### 10 *Characterization of the low voltage activated (LVA) $I_{\text{Ca}}$ current in myometrial smooth* 11 *muscle*

12 To determine the LVA  $I_{\text{Ca}}$  in freshly dissociated MSMCs we employed conventional  
13 patch clamp techniques combined with pharmacological maneuvers designed to separate  
14 the LVA and HVA  $I_{\text{Ca}}$ . Depolarizing pulses above -40mV elicited an inward current in all  
15 cells tested. In 54 of 98 cells tested, an inward current became apparent at voltages  
16 positive to -60mV indicating the presence of LVA  $I_{\text{Ca}}$ . In a number of control cells (n=12  
17 for HVA expressing cells and n=12 for HVA/LVA expressing cells) we utilized two  
18 current-voltage (I-V) protocols from a holding potential of -50mV and -80mV  
19 respectively to distinguish the LVA and HVA components. From the resultant I-V plots  
20 we determined the approximate contribution of LVA and HVA channels to the overall  
21 inward current (Fig 3 A,B,C). This analysis yielded a population of cells with HVA  
22 current (Fig 3A) and a different population of cells demonstrating both LVA and HVA  
23 current (Fig 3B). After determination of the LVA  $I_{\text{Ca}}$  by I-V plot at HP -50 and HP -80

1 we added 1  $\mu$ M nifedipine to remove the dihydropyridine sensitive component of the HVA  
2 current (Fig 3 D,E,F) (Fig 4A(a),(b)) and repeated the I-V protocol. This established that  
3 the peak LVA response in the presence of nifedipine could be estimated in a more rapid  
4 protocol by an assay for current density at -30mV from a holding potential of -80mV.  
5 Under these conditions LVA current was present in only 55% of cells tested (54/98 cells)  
6 which was in contrast to 100% (98/98 cells) for the HVA current. The mean cell  
7 capacitance of HVA expressing cells ( $140 \pm 15$  pF) was not significantly different to  
8 HVA/LVA expressing cells ( $142 \pm 13$  pF) ( $P > 0.05$ ).  
9 The LVA  $I_{Ca}$  was then further characterized using the divalent ion  $Ni^{2+}$  which has  
10 previously been demonstrated to block  $Ca_v3$  subunits.  $Ca_v3.2$  can be discriminated from  
11  $Ca_v3.1$  and  $Ca_v3.3$  by a 20-fold greater sensitivity to inhibition by  $Ni^{2+}$  (Lee *et al.*, 1999b).  
12 In agreement with the immuno-histochemistry data suggesting that  $Ca_v3.1$  is the  
13 predominant LVA  $I_{Ca}$  in MSMCs, the majority of cells with LVA  $I_{Ca}$  (53/54) tested were  
14 relatively insensitive to 120  $\mu$ M nickel. Furthermore, a dose dependent response to nickel  
15 (Fig. 4A(c), B) demonstrated an  $IC_{50}$  of  $118.57 \pm 68.9$   $\mu$ M ( $n=3$ ) at -30mV which is similar  
16 to that ( $167 \pm 15$   $\mu$ M) in *Xenopus* oocytes expressing  $Ca_v3.1$  (Lee *et al.*, 1999b). The  
17 inhibition of LVA current by nickel was voltage dependent with  $IC_{50}$  decreasing to  
18  $73.741 \pm 17.5$  at -40mV. In separate control experiments prior to nifedipine addition 100  
19  $\mu$ M nickel did not significantly inhibit HVA  $I_{Ca}$  (Fig. 4A(b)) (mean inhibition versus  
20 control at -30mV from -80mV holding potential =  $0.5\% \pm 0.01$  ( $n=3$ ),  $P > 0.05$ ).  
21 After establishing that the LVA  $I_{Ca}$  in human MSMCs was most likely to be  $Ca_v3.1$  we  
22 attempted to determine the physiological function of the current. The precise function of  
23 a LVA  $I_{Ca}$  in smooth muscle has remained the subject of much conjecture due to the

1 resting membrane potential lying near steady state inactivation of these channels. This  
2 property, could theoretically lead to the establishment of a “window” current at the  
3 resting membrane potential, which may either contribute to calcium dependent cellular  
4 processes or lead to a slow depolarisation. We therefore utilized a two-pulse protocol to  
5 measure the parameters of steady state activation and inactivation of both LVA and HVA  
6  $I_{Ca}$  in MSMCs to determine the “window” current. Cells were held at HP of -80mV  
7 followed by incremental pulses of 10mV for 500 msec (conditioning pulses) followed by  
8 re-polarization to -80mV and finally a 60 msec pulse to 0mV (test pulse). To evaluate the  
9 steady-state inactivation, peak currents recorded during the test pulse to 0mV after each  
10 conditioning pulse were normalized to a maximum and plotted as a function of the  
11 conditioning pulse amplitude. Steady-state activation was determined from the peak  
12 amplitude of inward current during conditioning pulses. The mean and standard error of  
13 6 cells expressing both HVA and LVA currents are shown in Figure 4C. Boltzmann fit  
14 revealed half-activation of inward current at  $-46 \pm 0.4$  mV with a slope factor of  
15 approximately 5 mV. Half-inactivation of inward current was observed at  $-47 \pm 0.5$  mV  
16 with 9mV slope factor. A pronounced overlap between steady-state activation and  
17 inactivation curves was evident, peaking at approximately -50 mV. At the reported  
18 resting membrane potential of approximately -55mV in human myometrium at  
19 term(Parkington *et al.*, 1999a) this overlap would lead to a persistent inward current.  
20 A well established role for  $Ca_v3.1$  in the central nervous system is to participate in burst  
21 firing whereby a combination of  $I_h$  and  $Ca_v3.1$  elicit repetitive depolarization through low  
22 threshold calcium spikes(Kim *et al.*, 2001) upon which fast sodium spikes are  
23 superimposed. In our hands, under standard current clamp conditions, there was no



1 repetitive firing of action potentials in human isolated myocytes, which is in contrast with  
2 both rat and mouse myocytes. A typical current clamp recording of isolated human MSM  
3 in response to 15 second 10pA depolarizing pulse is depicted in Fig. 5A. A depolarizing  
4 current pulse of 15 second duration resulted in a single action potential with a threshold  
5 around -40mV. However a subsequent 5 second hyper-polarization to a potential greater  
6 than -80mV lead to a rebound (anode break) action potential at a threshold of  
7 approximately -65mV. This rebound LVA spike was completely inhibited by 100μM  
8 nickel and recovered after subsequent washout.

9 Finally, we sought to establish the role of the LVA  $I_{Ca}$  current in contractions of freshly  
10 isolated MSM. A previous study in humans using the partly selective T-type inhibitor  
11 Mibefradil demonstrated an inhibition of contractile activity that was associated with a  
12 decrease in the magnitude of the initiation spike of the tissue level action potential  
13 (Young & Zhang, 2005). However, a number of studies have drawn into question the  
14 selectivity of Mibefradil (Viana *et al.*, 1997; Gomora *et al.*, 1999; Eller *et al.*, 2000) and  
15 subsequent conclusions about *in vivo* effects; most recently culminating in the  
16 demonstration of specific inhibition of HVA calcium channels in the cardiovascular  
17 system (Moosmang *et al.*, 2006). Given our electrophysiology data, and a reported  $K_i$   
18 value of 800μM (Zamponi *et al.*, 1996) for the HVA L-type subunit  $\alpha_{1C}$  (with calcium as  
19 the permeant ion) we utilized 100μM nickel to inhibit the LVA  $I_{Ca}$  in spontaneously  
20 contracting myometrial strips.

21 In these experiments addition of 100μM nickel increased mean contraction interval by  
22 3.5-fold over a 30-50 minute period of application ( $P < 0.05$ ;  $n = 4$ ) (Fig. 6A,C) an effect  
23 that was reversible on washout (Fig. 6B). In addition to an effect on contractile

frequency, a small but significant ( $P < 0.001$ ) increase was observed in activity integral, but there was no significant effect on maximum contractile force (Fig. 6C).

### Discussion

In this study we have investigated the expression of the three T-Type alpha sub-units,  $Ca_v3.1$ ,  $Ca_v3.2$ , and  $Ca_v3.3$  in human myometrium. Initially, we established that the  $Ca_v3.1$  and  $Ca_v3.2$  isoforms are expressed in human myometrial samples. This was in agreement with a previous study utilizing RT-PCR that demonstrated both  $Ca_v3.1$  and  $Ca_v3.2$  are differentially expressed throughout gestation in the different layers of rat myometrium(Ohkubo *et al.*, 2005b). In our study we used a panel of cDNA derived from patients at different times of gestation and labour status to establish if there were gestation related changes in  $Ca_v3$  isoforms. In contrast with the rat study we found no significant difference in expression in the  $Ca_v3$  isoforms with either gestation or with labour. Following RT-PCR analysis we sought to establish whether there were different types of cells expressing  $Ca_v3.1$  and  $Ca_v3.2$ . Utilizing immuno-histochemistry we found that  $Ca_v3.1$  was expressed in some MSMCs and in vascular smooth muscle cells. In contrast,  $Ca_v3.2$  demonstrated positive staining in vascular endothelial cells and leukocytes. This is the first description of the cellular distribution of  $Ca_v3$  isoforms in the human uterus and demonstrates that the expression of  $Ca_v3.2$  splice variants as described by Ohkubo *et al.*(Ohkubo *et al.*, 2005a) is unlikely to be physiologically relevant to smooth muscle cells but is more likely to be relevant to invading leukocytes. Interestingly, block of the T-type current in leukocytes has been demonstrated to inhibit adhesion (and subsequent invasion) to vascular endothelial cells by inhibiting the calcium dependent expression of  $\beta 2$ -integrins and L-selectin(Nebe *et al.*, 2002). Given the

1 importance of invading leukocytes in the process of parturition this may warrant further  
2 investigation.

3 We sought to characterize the function of the T-type current and establish the density of  
4  $\text{Ca}_v3.1$  current in myometrial cells by electrophysiology. Utilizing a combination of  
5 biophysical parameters, nifedipine and nickel, we were able to establish that there was  
6 LVA current in 55% of myometrial cells, which was in contrast to 100 % of cells  
7 demonstrating a HVA current. Our voltage-clamp data was in good agreement with that  
8 of Young et al (Young *et al.*, 1993) and Knock & Aaronson (Knock & Aaronson, 1999)  
9 who demonstrated that both LVA and HVA  $\text{Ca}^{2+}$  currents were present in freshly  
10 dissociated MSMCs.

11 The LVA current was blocked, in a voltage-dependent manner, by nickel with an  $\text{IC}_{50}$  at -  
12 30mV that is consistent with recordings made from oocytes expressing  $\text{Ca}_v3.1$  (Lee *et al.*,  
13 1999b). This further supported the immuno-histochemistry data suggesting that  $\text{Ca}_v3.1$  is  
14 the predominant isoform in MSM.

15 Our data suggests that LVA  $\text{I}_{\text{Ca}}$  is present in approximately 50% of cells which raises the  
16 question of whether there is a gestation dependent shift in the proportion of cells  
17 expressing LVA  $\text{I}_{\text{Ca}}$ ? Unfortunately we were unable to address this question by assessing  
18 current density per patient because it is not technically possible to assay a sufficient  
19 population of cells from any given patient. However, if the overall ratio of cells  
20 expressing LVA  $\text{I}_{\text{Ca}}$  was changing with gestation and assuming that mRNA reflects  
21 protein levels and that a single biopsy is a sample of millions of cells one would expect to  
22 see an overall change in expression of  $\text{Ca}_v3.1$  mRNA. Since this was not the case in our

1 study, and in the absence of good population current density data, we assume there to be  
2 no overall change in ratio.

3 The precise physiological role of the T-type calcium channel in smooth muscle remains  
4 the subject of debate (Perez-Reyes, 2004; Fry *et al.*, 2006). This is largely because,  
5 unlike some neurons where resting membrane potentials of -70 to -80mV and/or  
6 contributions of hyperpolarizing currents lead to LVA spikes from the T-type, smooth  
7 muscle cells have resting membrane potentials within the range for steady-state  
8 inactivation of the T-type channel. However, due to the particular biophysical properties  
9 of the channel there remains a possibility that a small population of channels may  
10 contribute to a window current (Perez-Reyes, 2003) at the resting membrane potential of  
11 the smooth muscle cell and thereby contribute to either a slow wave depolarisation or  
12 other calcium-dependent intracellular processes. To elucidate the potential for both a  
13 contribution to a LVA spike or window current we undertook a combination of voltage  
14 clamp and current clamp experiments. Voltage-clamp experiments to determine the  
15 steady state activation and inactivation kinetics of the combined LVA and HVA inward  
16 currents demonstrated a window current between -60mV to 0mV. This is particularly  
17 interesting since the resting membrane potential of the myometrium becomes steadily  
18 depolarized throughout gestation from -80mV at mid-gestation to -55mV at labour and  
19 delivery (Parkington *et al.*, 1999a). At term therefore, due to the resting membrane  
20 potential being within the window current, a slow depolarisation or calcium “leak” may  
21 occur within smooth muscle cells expressing the T-type channel.

22 To explore the possibility of the T-type mediating a LVA spike in myometrial cells we  
23 tested cells under current clamp conditions by an initial depolarization to ensure the

1 initiation of an action potential followed by a hyper-polarization to negative potentials  
2 sufficient to de-inactivate the T-type. Consistent with the classical role of the T-type,  
3 hyper-polarization (to greater than -80mV) was followed by a rebound LVA spike that  
4 was abolished by nickel and recovered upon wash out. This suggests that  $Ca_v3.1$  in  
5 MSMCs can elicit LVA spikes although it is unclear as to what the identity of the  
6 endogenous hyperpolarizing current might be that could drive de-inactivation. The  
7 physiological role of this process requires further investigation.

8 If there is a slow depolarisation via the T-type current in MSMCs, or that a steady  
9 calcium leak contributed to the activation of a calcium dependent inward conductance,  
10 one would hypothesize that an inhibition of this current would slow the frequency of  
11 contractions since any slow depolarisation/activation of an inward calcium conductance  
12 would increase the chances of a threshold event. Utilizing a concentration of nickel  
13 consistent with specific block of the T-type and not L-Type current in this study and well  
14 below the  $K_i$  (800 $\mu$ M) for recombinant L-type subunit  $\alpha_{1C}$  (Zamponi *et al.*, 1996) we  
15 determined that the frequency of spontaneous contractions of myometrial strips was  
16 significantly reduced. Furthermore, the effect was reversible on wash out and caused no  
17 significant decrease in maximal contractile force. We observed a small but significant  
18 increase in activity integral that was due to an increase in duration of contraction.

19 Whether this was a direct result of T-type blockade or an effect due to reduced  
20 contraction frequency remains to be determined. It should be noted that there are a  
21 number of plausible hypotheses other than slow depolarisation that may lead to the T-  
22 type channel affecting contraction frequency. For example, a steady calcium leak may  
23 contribute to a slow filling of the sarcoplasmic reticulum, the filling status of which, will

1 determine spontaneous release and subsequent activation of the calcium sensitive  $I_{Cl}$   
2  $(Ca)$  current, previously demonstrated to affect contraction frequency (Jones *et al.*, 2004).  
3 The effect of nickel on other conductances that may contribute to contraction frequency  
4 cannot be ruled out. However, the concentration of nickel used in this study are well  
5 below the millimolar concentrations required for block of Na/Ca exchange or Na-K ATP-  
6 ase and addition of ouabain actually increases contraction frequency (Parkington *et al.*,  
7 1999b) suggesting a non-specific effect on a sodium pump is unlikely.  
8 We conclude that the primary T-type subunit expressed in some MSMCs is  $Ca_v3.1$ . We  
9 show that the LVA  $I_{Ca}$  conductance is heterogeneously expressed in human myometrial  
10 smooth muscle cells and that cells expressing both LVA  $I_{Ca}$  and HVA  $I_{Ca}$  demonstrate an  
11 extended window current. We found that 100  $\mu$ M nickel reduces the contraction  
12 frequency of spontaneously contracting human myometrium.

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5

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## Figure Legends.

**Fig 1.** mRNA expression of CAV3.1 (Panel A) and CAV3.2 (Panel B) in myometrial samples taken from patients Preterm not in labour (PTNIL; circle), Preterm in labour (PTL; square), Term not in labour (NIL; triangle), Term labour (TL, Inverted triangle). Each point represents individual patients normalized by  $2^{-\Delta\Delta CT}$  method to r18S and non-pregnant myometrium. Patients group means were not significantly different  $P > 0.05$  One-way ANOVA (Neuman-Keuls) test.

**Fig. 2.** Immunohistochemistry for  $Ca_v3.1$  &  $Ca_v3.2$ . Channels were visualized by confocal microscopy using secondary antibodies conjugated to Alexa 633 and are depicted in red. Nuclei were counterstained with DAPI and are depicted in blue.  $Ca_v3.1$  (Alexa 633, Red; Nuclei DAPI, Blue) localised to myometrial smooth muscle cells (Panel A, bar  $50\mu M$ ) and vascular smooth muscle cells (Panel D, bar  $50\mu M$ ) but not leukocytes (Panel G, bar  $50\mu M$ ) in myometrium.  $Ca_v3.2$  (Alexa 633, Red; DAPI, Blue) by contrast did not localize to myometrial smooth muscle cells (Panel B, bar  $50\mu M$ ) but to vascular endothelium (Panel E, bar  $50\mu M$ ) and leukocytes (Panel H, bar  $50\mu M$ ). Panel C, F, & I correspond to negative control (Secondary Ab only). In isolated myocytes detailed scans localized  $Ca_v3.1$  to punctate regions of the plasma membrane and the sarcoplasmic reticular region (Panel J, Bar  $20\mu M$ ). For contrast F-Actin cytoskeleton is highlighted by Phalloidin-488 (green) (Panel K) and Panel J & K are presented in overlay in Panel L.

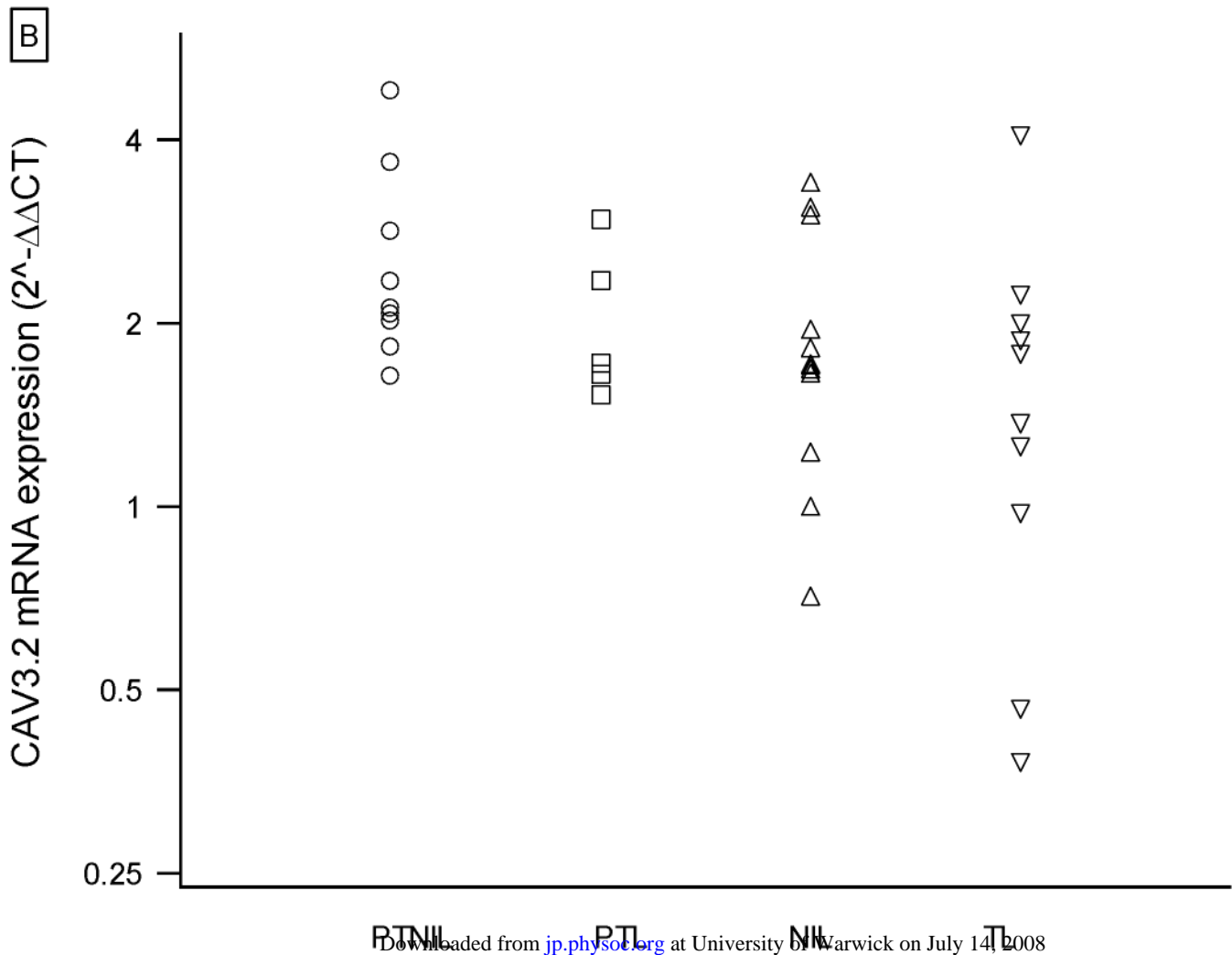
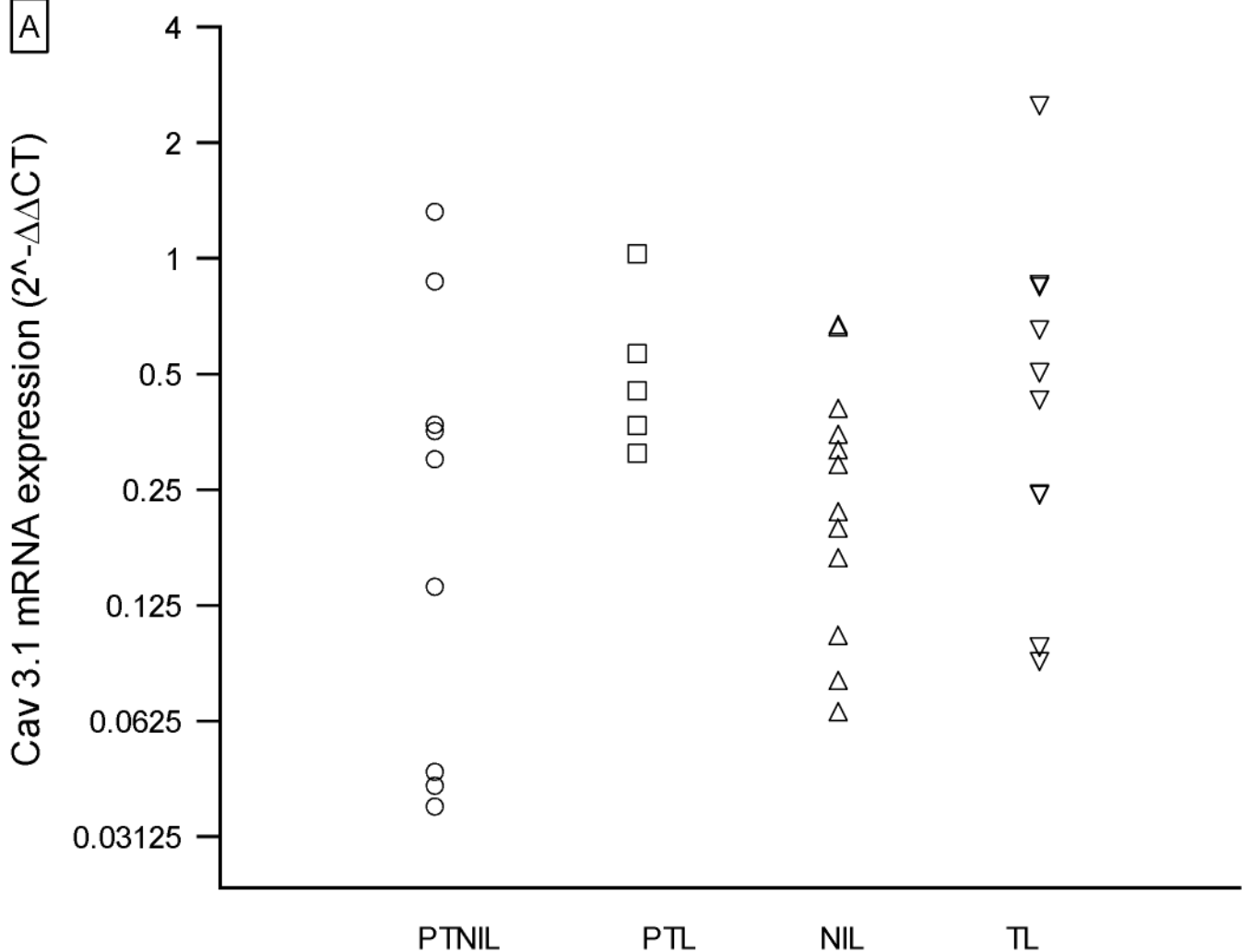
**Fig. 3.** Current-voltage relationship of  $I_{Ca}$  in HVA and HVA/LVA expressing cells. Panel A. HVA expressing cells (mean  $\pm$  SE (n=12 per point)) current density vs step voltage from a holding potential of  $-50mV$  (closed circle) and  $-80mV$  (closed square). Panel B HVA/LVA expressing cells (mean  $\pm$  SE (n=12 per point)) current density vs step voltage from a holding potential of  $-50mV$  (closed circle) and  $-80mV$  (closed square). Panel C. Mean current density at holding potential  $-50mV$  subtracted from mean current density at holding potential  $-80mV$  vs step voltage. Subtracted current density of HVA expressing cells (closed circle) and HVA/LVA expressing cells (closed square) are significantly different at step potentials of  $-50$ ,  $-40$ ,  $-30$  and  $-20mV$  only. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Panel D. HVA expressing cells (mean  $\pm$  SE (n=4 per point)) current density vs step voltage from a holding potential of  $-50mV$  in the absence (closed circle) and presence (closed square) of  $1\mu M$  nifedipine. Panel E. HVA/LVA expressing cells (mean  $\pm$  SE (n=4 per point)) current density vs step voltage from a holding potential of  $-50mV$  in the absence (closed circle) and presence (closed square) of  $1\mu M$  nifedipine. Panel F. Panel E. HVA/LVA expressing cells (mean  $\pm$  SE (n=4 per point)) current density vs step voltage from a holding potential of  $-80mV$  in the absence (closed circle) and presence (closed square) of  $1\mu M$  nifedipine.

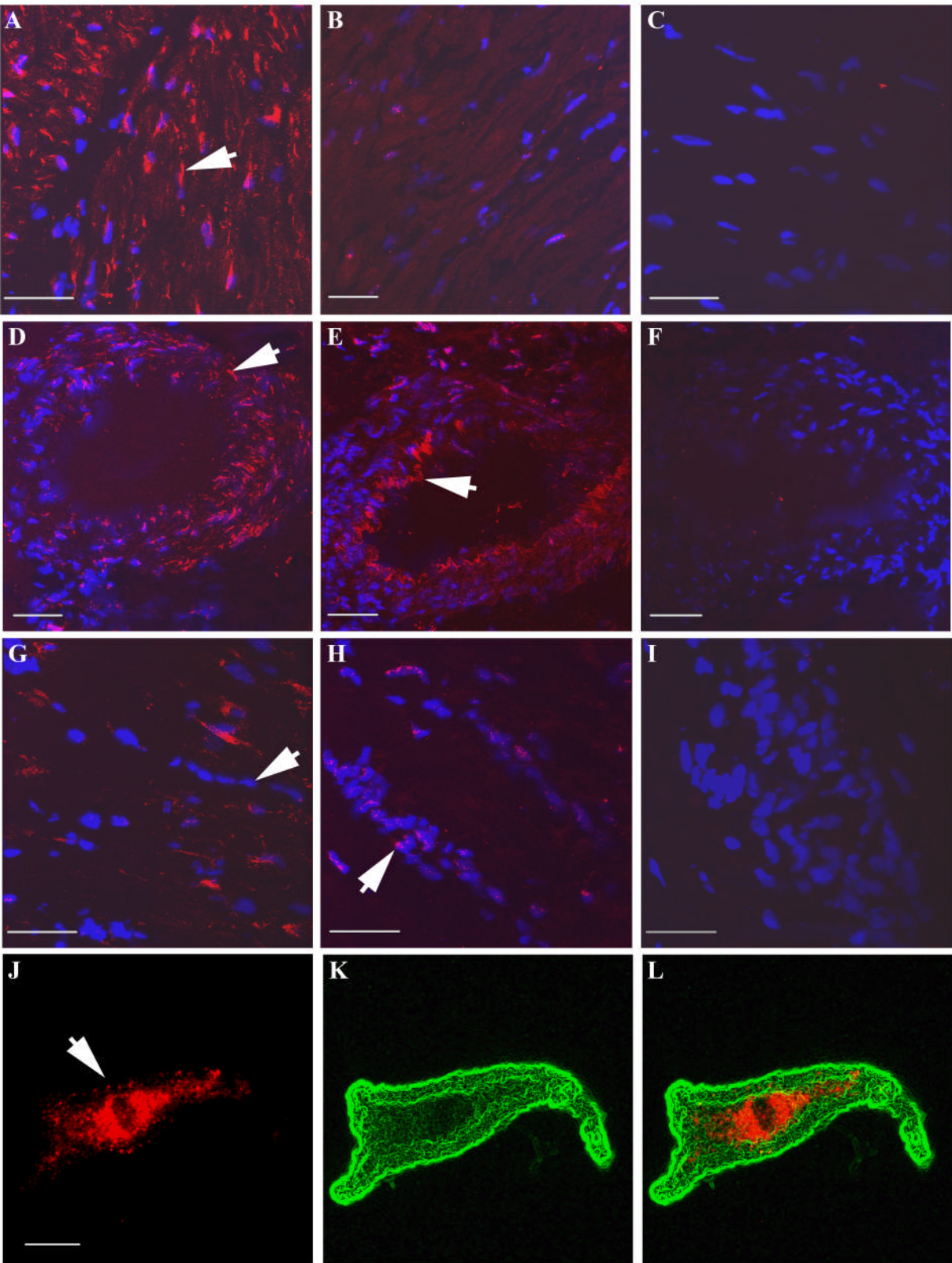
**Fig. 4.** Panel A. Effect of nifedipine and nickel on HVA and LVA  $I_{Ca}$ . Aa. Effect of increasing concentrations of nifedipine on LVA  $I_{Ca}$  elicited by voltage step to  $0mV$  from  $-50mV$  holding potential in a HVA expressing cell. Ab. Effect of

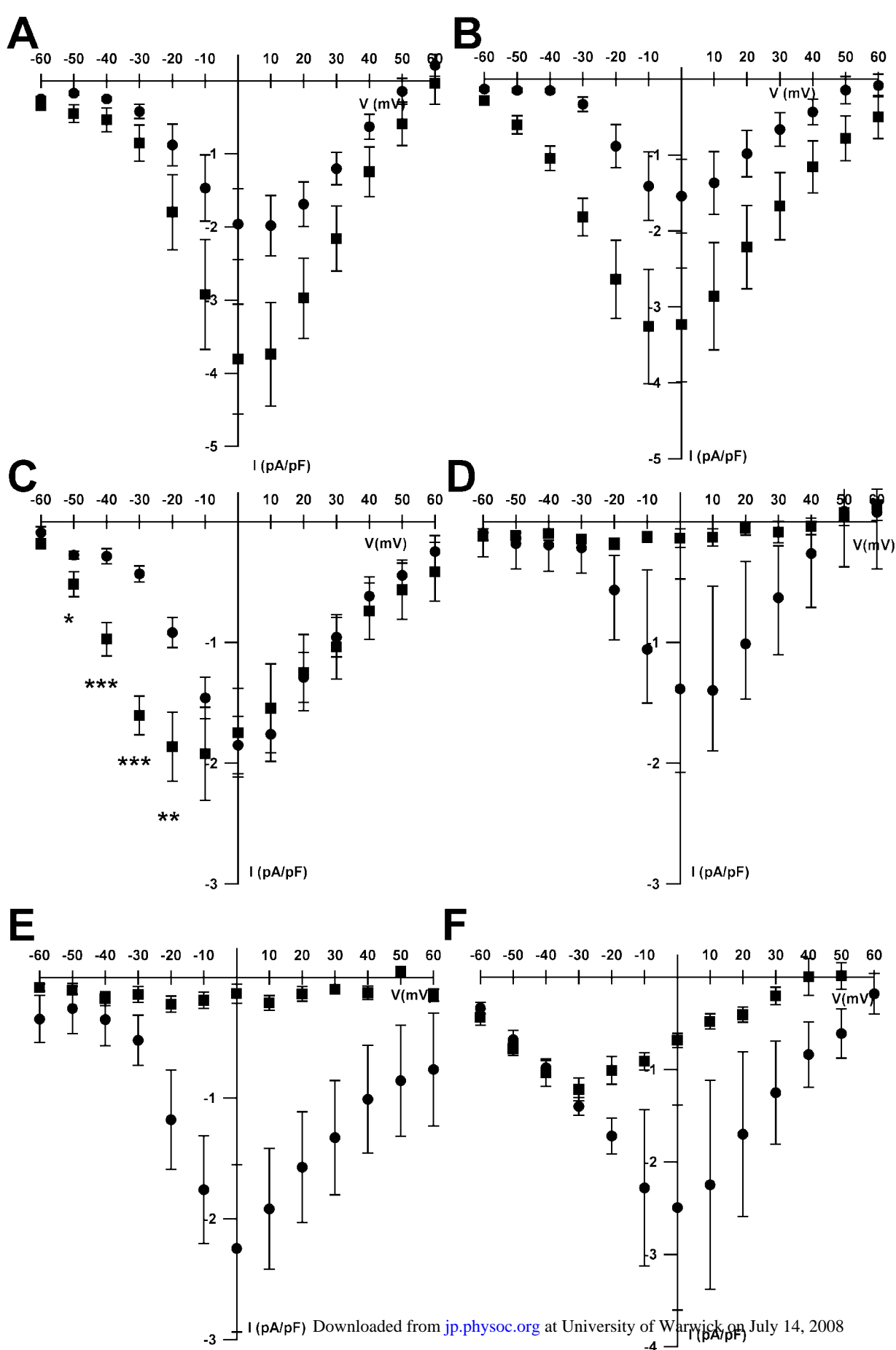
100 $\mu$ M nickel followed by 1 $\mu$ M nifedipine on HVA  $I_{Ca}$  elicited by voltage step to 0mV from -50mV holding potential in a HVA/LVA expressing cell. Ac. Effect of 1 $\mu$ M nifedipine followed by increasing concentrations of nickel on LVA  $I_{Ca}$  elicited by voltage step to -30mV from -80mV in a HVA/LVA expressing cell. Panel B. Dose dependent inhibition of LVA  $I_{Ca}$  current by nickel chloride. Values represent mean $\pm$ SE of percentage maximum current (n=3) Panel C. Steady state activation/inactivation curves determined from cells expressing both LVA and HVA current. Values represent mean $\pm$ SE (n=6). Curves were fitted using the standard Boltzmann function.

**Fig. 5.** Panel A. Current clamp trace depicting a single action potential response to a 15 second 10 pA depolarizing pulse followed by an LVA spike in response to a 5 second 10 pA hyperpolarizing pulse. Panel B. Time-scale expansion of the hyperpolarizing part of the protocol depicted in panel A. LVA spikes are elicited at approximately -65mV after a hyperpolarizing pulse and can be reversibly blocked by 100 $\mu$ M nickel chloride.

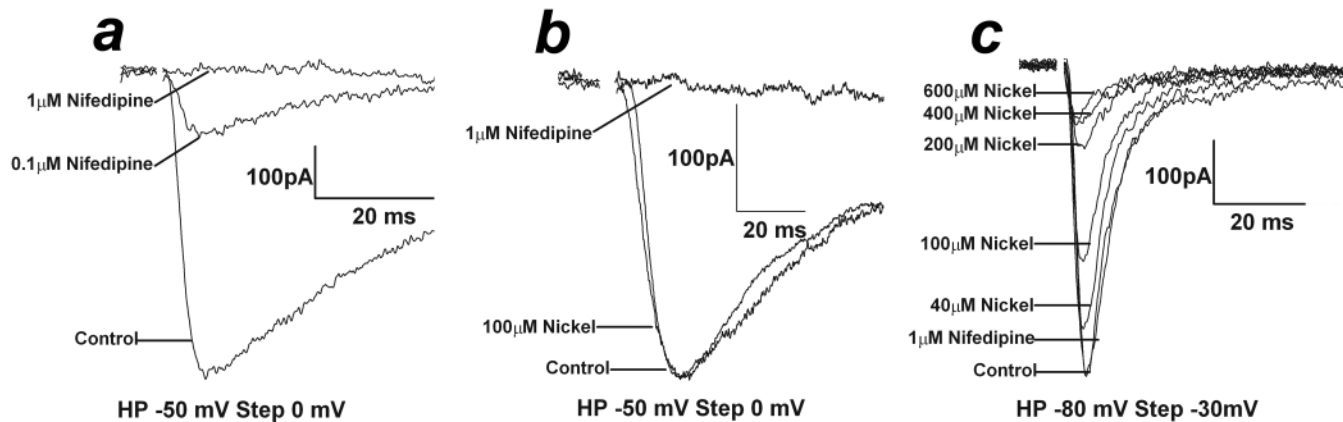
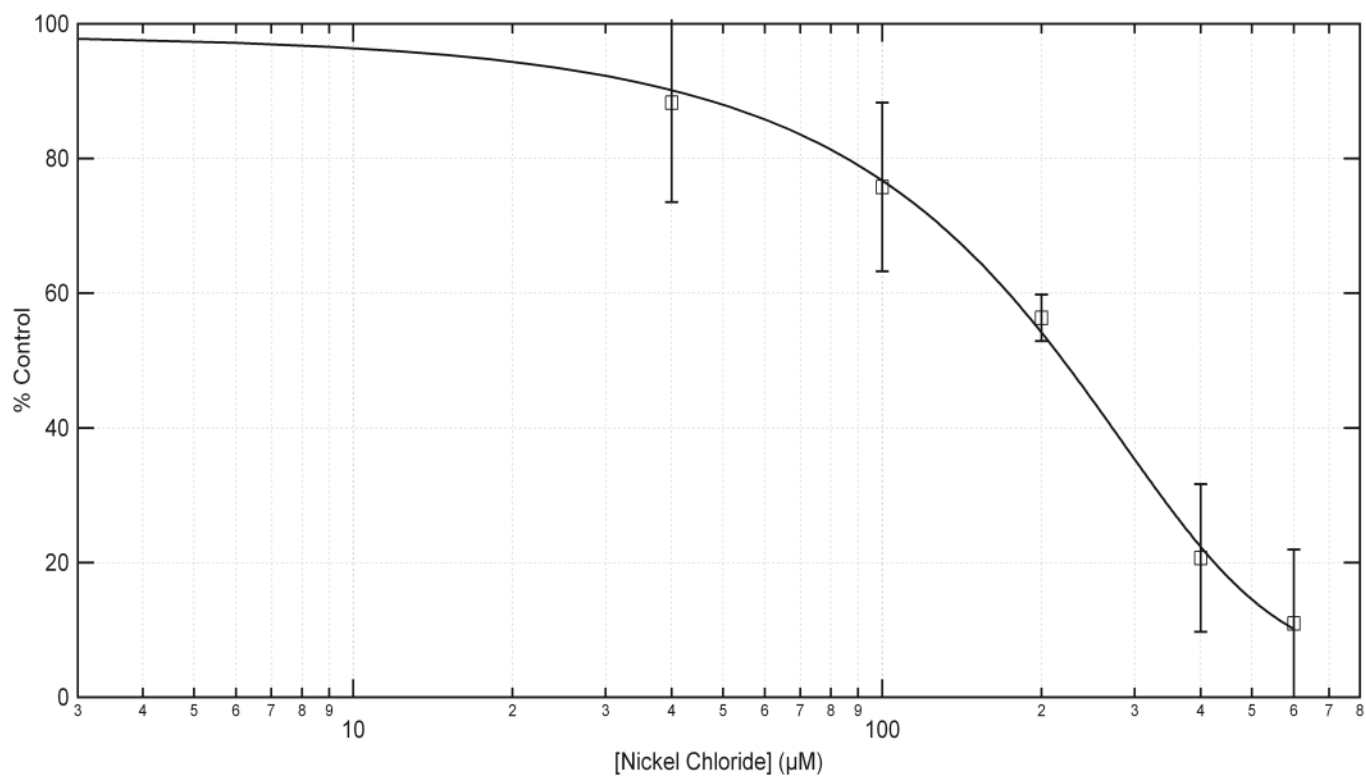
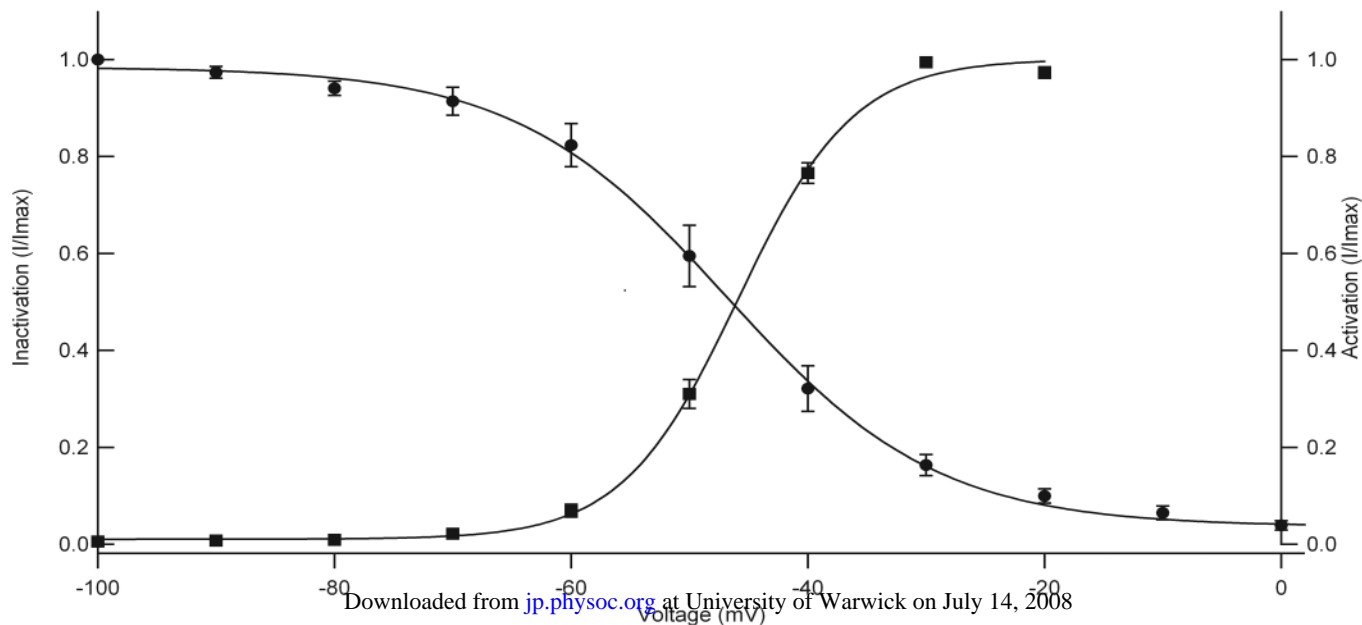
**Fig. 6.** Panel A. Spontaneous contractions of myometrial strips are slowed in frequency by application of 100  $\mu$ M nickel chloride. Panel B. Effect on frequency is gradual in onset and reversible on washout. (100  $\mu$ M nickel chloride; circle) (Time matched vehicle control; square). Panel C. Effect of 100 $\mu$ M nickel chloride on maximal contractile force (M-M), activity integral (AI) and contraction interval (CI). Values represent mean $\pm$ SE (n=4).  $P<0.05 = *$ ;  $P<0.001=***$



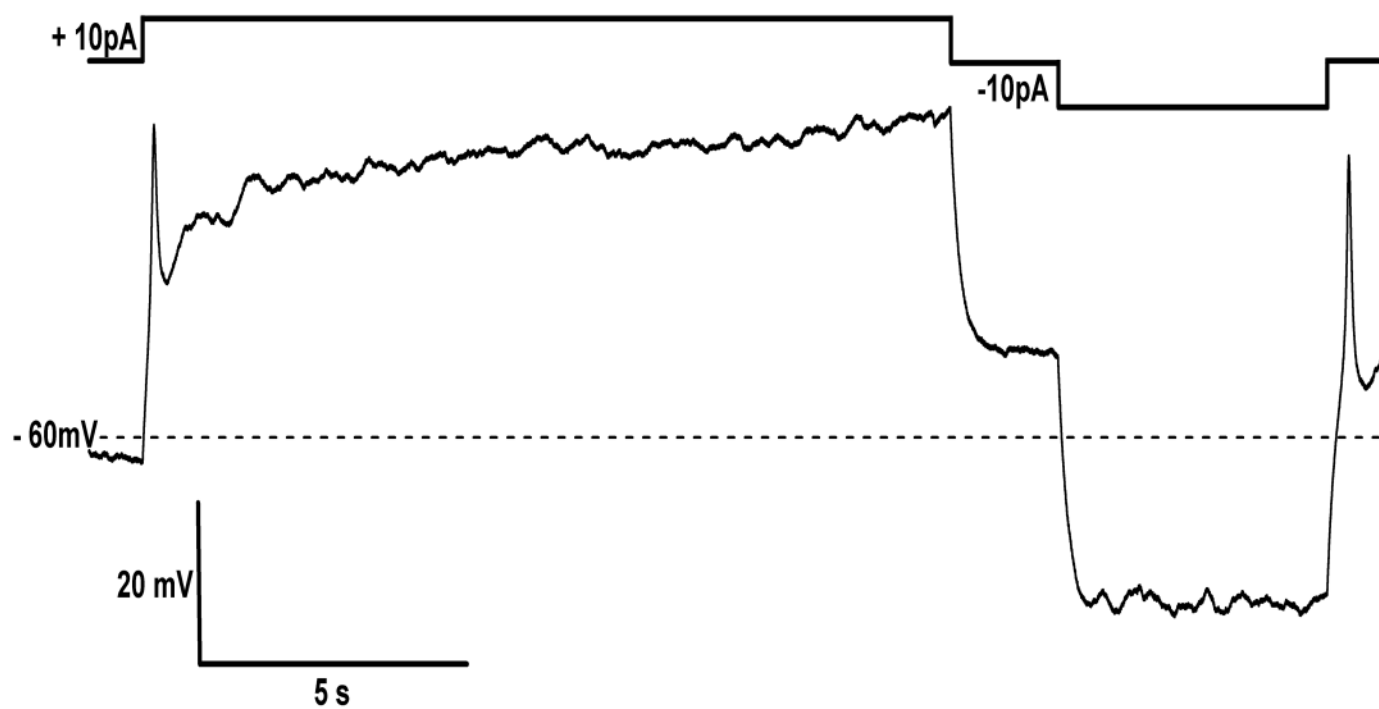
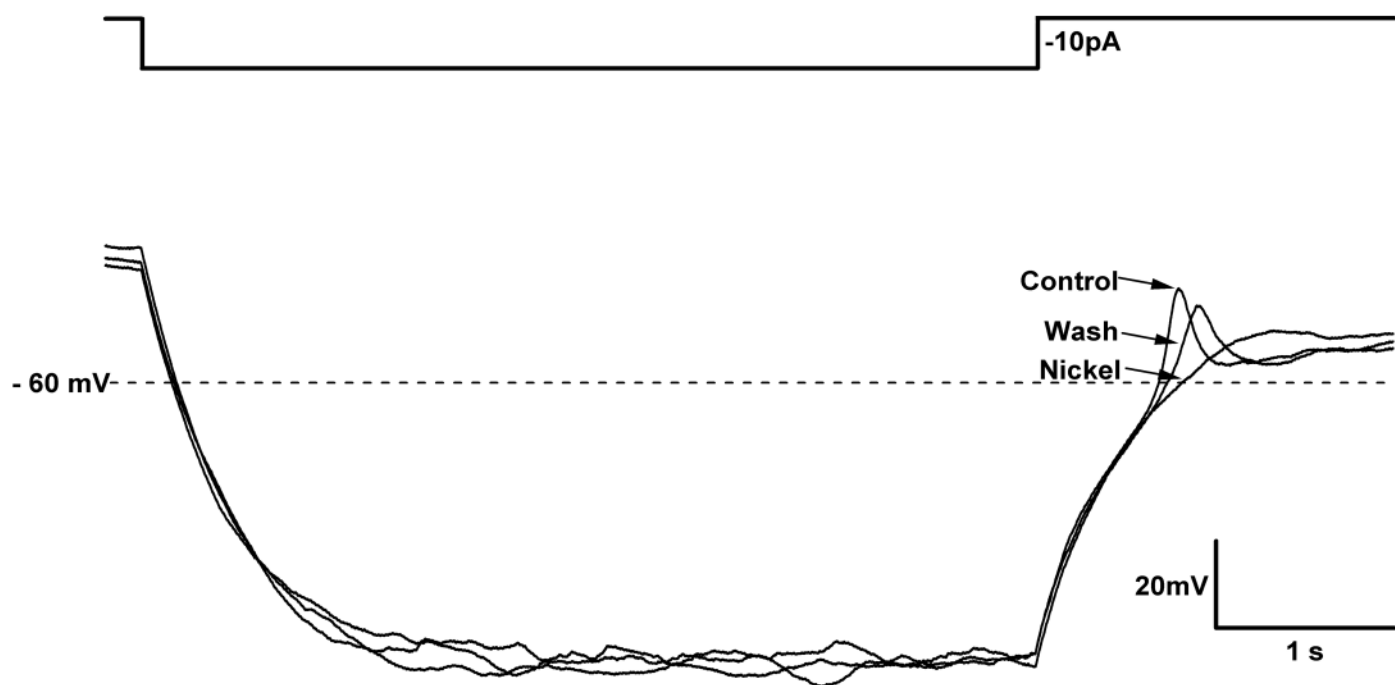


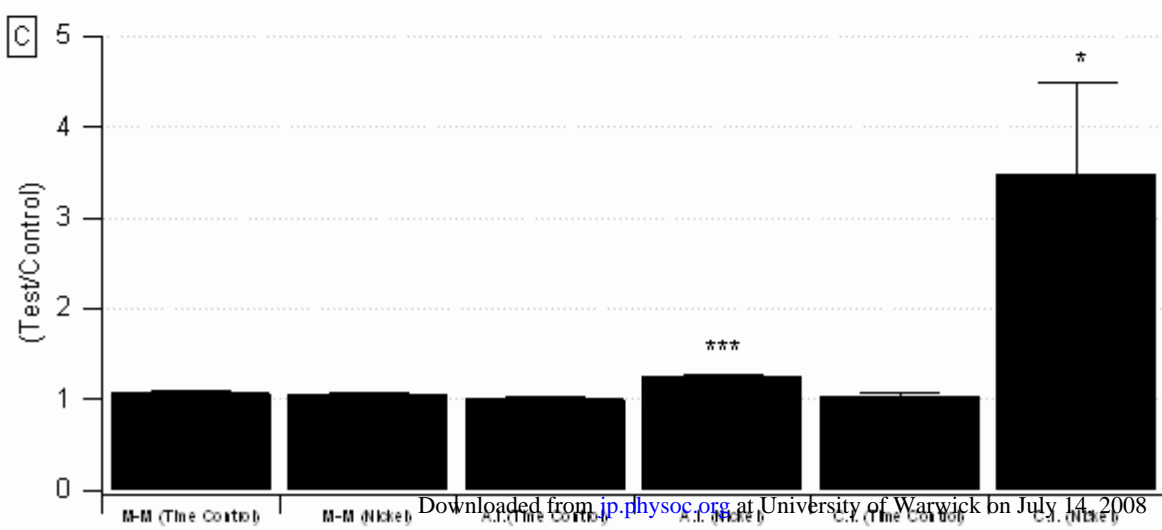
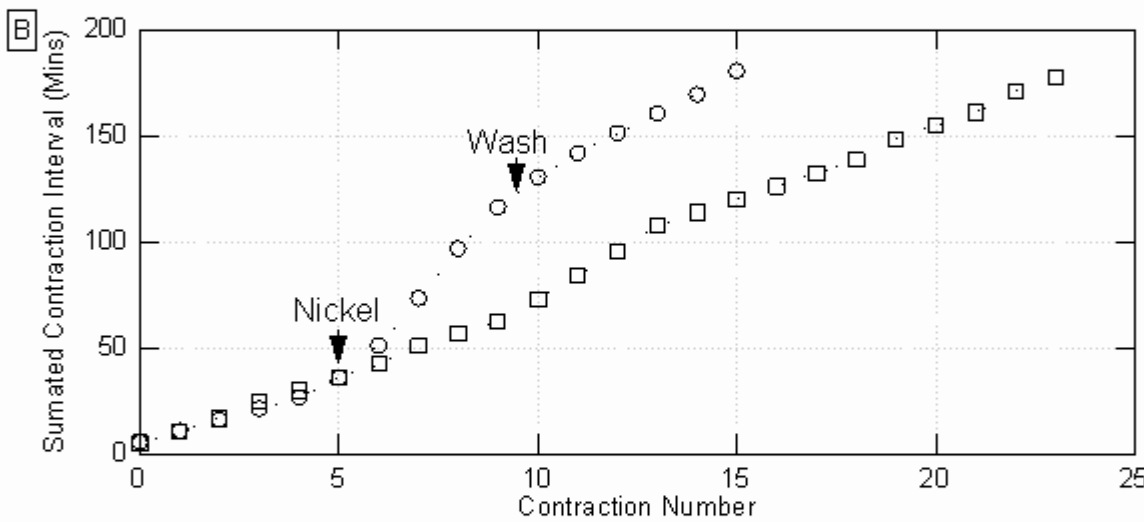
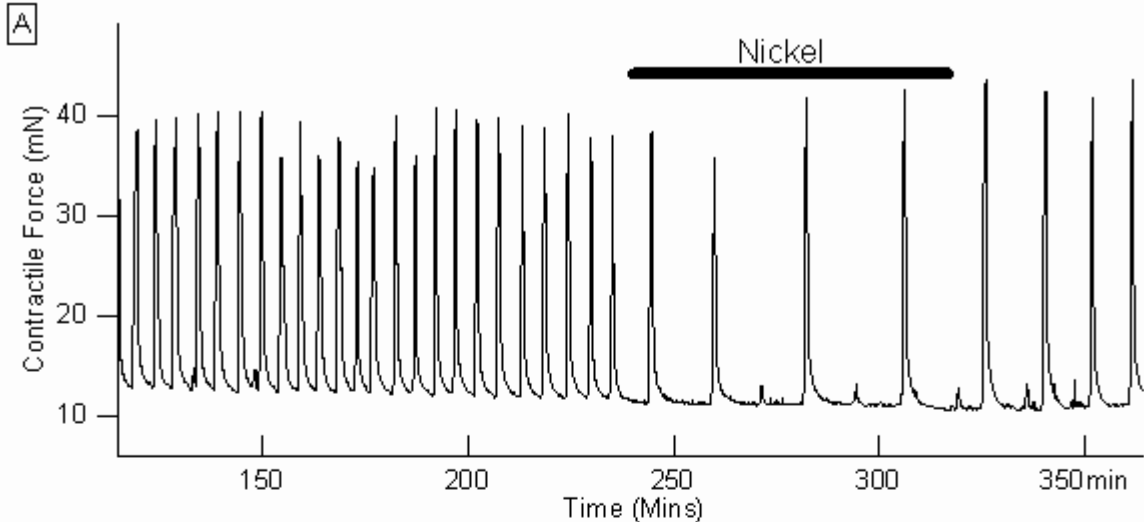




**A****B****C**



**A****B**



**Characterization of the molecular and electrophysiological properties of the  
T-Type calcium channel in human myometrium**  
Andrew M Blanks, ZhengHang Zhao, Anatoly Shmygol, Gilles Bru-Mercier, Shirley  
Astle and Steve Thornton

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